Cell Calcium 60 (2016) 1–12

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Steroid hormone 20-hydroxyecdysone promotes higher calcium mobilization to induce apoptosis

Di Wang, Xu-Yang Pei, Wen-Li Zhao, Xiao-Fan Zhao*

Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Sciences, Shandong University, Jinan 250100, China

ARTICLE INFO

Article history:
Received 4 February 2016
Received in revised form 11 April 2016
Accepted 4 May 2016
Available online 7 May 2016

Keywords:
20-Hydroxyecdysone
Juvenile hormone
Calcium
Calcium channels
Apoptosis

ABSTRACT

Calcium ions are essential secondary messengers that regulate diverse cellular processes including gene transcription, cell proliferation, and apoptosis. The steroid hormone 20-hydroxyecdysone (20E) promotes programmed cell death during insect metamorphosis, whereas juvenile hormone (JH) counteracts 20E activity to prevent metamorphosis. Both 20E and JH can induce cellular calcium increase; however, the mechanisms and physiological consequences resulting from calcium increase caused by the two counteracting hormones are unclear. Here, using *Helicoverpa armigera* epidermal cell line, we show that 20E via a G-protein-coupled receptor induced a major calcium rise in the cells, whereas JH via receptor tyrosine kinase induced a minor calcium increase. The calcium release-activated calcium modulator 1 (Orai1) and *transient receptor potential* (TRP) channels were necessary for 20E-induced rapid calcium influx. A higher calcium level was maintained in a long time and more genes including *Orai1* and *TRP* channels showed elevated expression after the treatment of 20E than did after JH treatment. Caspase3/7 activation, cell death and pro-apoptotic gene expression were elicited by 20E induction, but not by JH. JH could repress 20E-induced calcium influx, caspase3/7 activation and gene expression. Higher calcium levels induced apoptosis. These results suggest that 20E and JH via different pathways regulate calcium mobilization and homeostasis at different levels, thus inform different gene expression and cellular responses.

1. Introduction

Calcium is an important cellular secondary messenger, which regulates many different cellular processes via various intracellular signal pathways [1]. Changes in cytosolic free Ca^{2+} levels influence gene transcription [2], cell proliferation and cell death [3]. Small changes in cytosolic free Ca^{2+} can determinate the fate of T cells, including proliferation and apoptosis [4]. Cellular Ca^{2+} overload or perturbation of intracellular Ca^{2+} compartmentalization triggers either apoptotic or necrotic cell death [5]. In astrocytes, the homozygous type 2 inositol triphosphate receptor (IP_3R_2)-mediated Ca^{2+} signaling pathway ameliorates neuronal death and brain damage [6]. Therefore, calcium mobilization and homeostasis in cells are important for a number of cellular functions [7].

The free Ca^{2+} are controlled at a very low levels in the cells, about 10 ~ 100 nM, compared with mM calcium levels in the extracellular environment [8]. Extracellular signals trigger calcium ions mobilization, including intracellular Ca^{2+} release from endoplasmic reticulum (ER) storage, and extracellular Ca^{2+} influx from outside of the cells. Store-operated calcium entry (SOCE) is one of the major mechanisms of calcium mobilization. The intracellular calcium ions are released from ER-stored calcium under signal stimulation [9]. The depletion of ER calcium ions drives stromal interaction molecule 1 (STIM1) oligomerization and translocation from the ER to the cell membrane. STIM1 interacts with calcium release-activated calcium modulator 1 (Orai1) channels and/or transient receptor potential (TRP) cation channels to trigger extracellular calcium influx to increase cytosolic calcium levels [10]. By contrast, higher levels of calcium ions in the cells are excluded from the cytosol out of the cells via the plasma membrane calcium/calmodulin-dependent ATP enzyme (PMCA) [11], or stored in ER by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) to decrease cytosolic calcium levels [12].

Animal steroid hormones induce calcium mobilization through G-protein-coupled receptors (GPCRs). In humans, estrogen binds to GPCR30 (GPR30/GPER) to induce rapid intracellular calcium mobilization for gene expression and cell proliferation [13]. In the

Abbreviations: 20E, 20-hydroxyecdysone; JH, juvenile hormone; HaEpi, *Helicoverpa armigera* epidermal cell line; GPCR, G-protein-coupled receptor; RTK, receptor tyrosine kinase; STIM1, stromal interaction molecule 1; Orai1, calcium release-activated calcium modulator 1; TRP, channels and/or transient receptor potential; PMCA, plasma membrane calcium/calmodulin-dependent ATP enzyme; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase.

* Corresponding author.
E-mail address: xfzhao@sdu.edu.cn (X.-F. Zhao).

http://dx.doi.org/10.1016/j.ceca.2016.05.003
0143-4160/

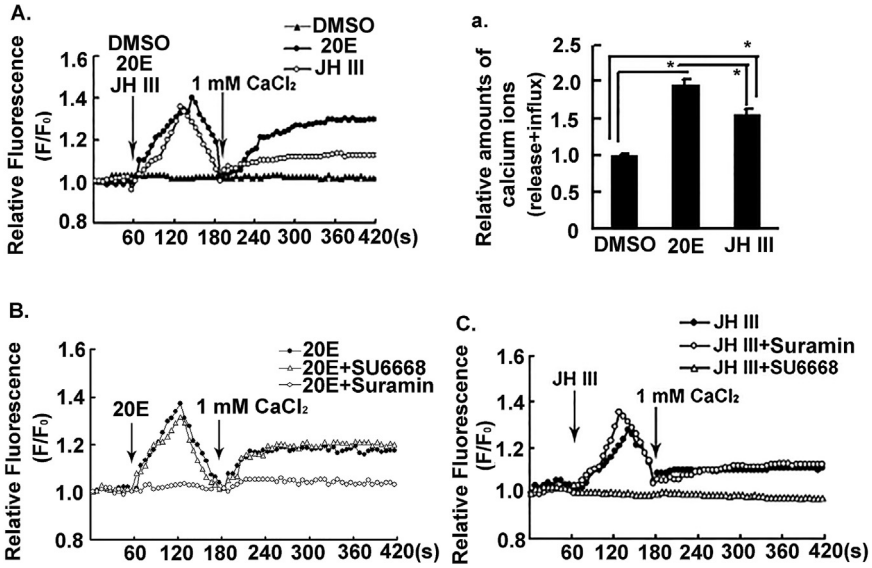

Fig. 1. 20E and JH III induce rapid intracellular calcium release and influx in HaEpi cells.
(A) 20E and JH III induced rapid cytosolic Ca^{2+} increase. Cells were treated for 1 h with AM ester calcium crimson™ dye (3 μM) in DPBS and then by 1 μM 20E, 1 μM JH III or 1 mM $CaCl_2$, or by an equal volume of DMSO as solvent control. Fluorescence was recorded using a Confocal Microscope at 555 nm and then analyzed using Image Pro-Plus software. F: fluorescence of cells after treatment; F_0: average fluorescence of cells before treatment. a. Statistical analysis of A. (B) Effect of GPCR inhibitor Suramin and RTK inhibitor SU6668 on the 20E-induced increase in Ca^{2+} levels. Suramin (50 μM) and SU6668 (5 μM) were added to the medium 30 min before 1 μM 20E induction at 27 °C. (C) Effect of SU6668 and Suramin on the 1 μM JH III-induced increase in Ca^{2+} levels, by the same treatments as in B.

Bombyx mori anterior silk gland, 20-hydroxyecdysone (20E), via unknown GPCRs, increases the intracellular Ca^{2+} level for apoptosis [14]. In *Helicoverpa arimigera*, 20E can induce rapid intracellular calcium release and extracellular calcium influx via ecdysone-responsive GPCRs (ErGPCRs) for metamorphosis [15,16]. 20E via ErGPCRs regulates phospholipase C-gamma-1 (PLCG1) activation to increase cellular calcium to regulate 20E pathway gene expression and metamorphosis [17]. The increased calcium activates calcium/calmodulin-dependent protein kinase II (CaMKII) to regulate its heterodimeric partner (USP1) via lysine acetylation for 20E pathway gene expression [18], including transcription factors *BrZ7* [19] and *HHR3* [20], for metamorphosis. The apoptosis-related genes protein kinase *prodeath-S/TK* [21] and *caspase1* [22] are then expressed for cell apoptosis.

The sesquiterpenoid juvenile hormone (JH) counteracts 20E activity to coordinate insect development, growth, reproduction and aging in insects [23,24]. The functions of 20E and JH III are quite different. 20E mainly initiates molting and metamorphosis in insects [25]. 20E triggers the apoptosis and autophagy of midgut cells during metamorphosis in *Drosophila* [26]. However, JH III prevents 20E-induced metamorphosis by modulating the activity of 20E [27]. A low cytosolic Ca^{2+}-level is hypothesized as the basis of the status quo effect of JH III [28]. A recent finding suggested that JH III induces calcium mobilization via receptor tyrosine kinase (RTK) in *Aedes aegypti*, [29]. The consequences and mechanisms of 20E-induced and JH-induced calcium mobilization and homeostasis are unclear.

In this study, we used *Helicoverpa armigera* epidermal cells (HaEpi) [30] as experimental material to detect calcium mobilization and homeostasis under 20E and JH induction, respectively. Our results confirmed that 20E, via GPCRs, and JH III, via RTKs, increased intracellular calcium release. However, 20E induced the expressions of more calcium ion channels to mobilize more extracellular calcium influx, finally maintaining calcium homeostasis at a higher level, which led to apoptosis. JH III induced the expressions of fewer calcium ion channels and thus mobilized lower extracellular calcium influx, resulting in a lower level of calcium homeostasis, which did not lead to apoptosis. Thus, two hormones, via different mechanisms, mobilized calcium and maintained calcium at different homeostasis levels to determine cell fate.

2. Materials and methods

2.1. Materials

An *Helicoverpa armigera* epidermal cell line (HaEpi) was established in our laboratory, as previously described [30]. HaEpi cells were cultured in Grace's medium (6.7 mM $CaCl_2$) with 10% fetal bovine serum (FBS, MDgenics, St. Louis, MO, USA) at 27 °C.

2.2. Cellular calcium ions examination

When the cell density reached 2×10^6, the cells were incubated with 3 mM acetoxymethyl (AM) ester calcium crimson™ dye (Invitrogen, Carlsbad, CA, USA) in Dulbecco's phosphate buffered saline (DPBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4) for 30 min at 27 °C, based on the above protocol. Cells were washed thrice with DPBS without calcium ions and then treated with 1 μM 20E to detect the calcium release from the ER to the cytosol (experiments were performed using different concentrations of 20E). After 60s, calcium chloride (1 mM) was added to the medium, because at 60s, hormone-induced calcium release from the ER was evacuated completely [31]. Fluorescence was detected at 555 nm every 6 s for 360 s (experiments detecting different length of time were also performed) using Carl Zeiss LSM 700 laser scan Confocal Microscope (Thornwood, NY, USA). The data were analyzed using Image Pro-Plus software (Media Cybernetics, USA). After opening the file for editing, the picture was converted into grayscale 8 for automatic counting measurements, and the measurement parameters were selected. Finally, the file was imported into Excel. The Excel file was then submitted for statistical analysis. For the interference experiments, the cells were incubated with *dsRNA* for 24 h as previously described. For the inhibition experiments, the cells were pretreated with different inhibitors for 30 min at 27 °C before washing thrice with DPBS without calcium ions and stimulation with 20E. Suramin (Sigma,